Biochimica et Biophysica Acta 1853 (2015) 361–369

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Downregulation of diacylglycerol kinase ζ enhances activation of cytokine-induced NF-κB signaling pathway

Rieko Tsuchiya [a,b], Toshiaki Tanaka [a], Yasukazu Hozumi [a], Tomoyuki Nakano [a], Masashi Okada [a], Matthew K. Topham [c], Mitsuyoshi Iino [b], Kaoru Goto [a,*]

[a] Department of Anatomy and Cell Biology, Yamagata University School of Medicine, Yamagata 990-9585, Japan
[b] Department of Dentistry, Oral and Maxillofacial Plastic and Reconstructive Surgery, Yamagata University School of Medicine, Yamagata 990-9585, Japan
[c] Huntsman Cancer Institute, Department of Oncological Sciences, University of Utah, Salt Lake City, UT 84112, USA

ARTICLE INFO

Article history:
Received 11 August 2014
Received in revised form 31 October 2014
Accepted 10 November 2014
Available online 15 November 2014

Keywords:
Diacylglycerol kinase ζ
Inflammation
TNF-α
NF-κB
Nuclear translocation
CBP

ABSTRACT

The transcription factor NF-κB family serves as a key component of many pathophysiological events such as innate and adaptive immune response, inflammation, apoptosis, and oncogenesis. Various cell signals trigger activation of the regulatory mechanisms of NF-κB, resulting in its nuclear translocation and transcriptional initiation. The diacylglycerol kinase (DGK) family, a lipid second messenger-metabolizing enzyme in phosphoinositide signaling, is shown to regulate widely various cellular processes. Results of recent studies suggest that one family member, DGKζ, is closely involved in immune and inflammatory responses. Nevertheless, little is known about the regulatory mechanism of DGKζ on NF-κB pathway in cytokine-induced inflammatory signaling. This study shows that siRNA-mediated DGKζ knockdown in HeLa cells facilitates degradation of IκB, followed by nuclear translocation of NF-κB p65 subunit. In addition, DGKζ-deficient MEFs show upregulation of p65 subunit phosphorylation at Serine 468 and 536 and its interaction with CBP transcriptional coactivator upon TNF-α stimulation. These modifications of p65 subunit might engender enhanced NF-κB transcriptional reporter assay of DGKζ knockdown cells. These findings provide further insight into the regulatory mechanisms of cytokine-induced NF-κB activation.

1. Introduction

The transcription factor nuclear factor-κB (NF-κB) plays a central role in regulating inflammation, immune responses, cell growth and survival, and tumorigenesis [1,2]. It is involved in various human diseases and is targeted by several anti-inflammatory and anticancer drugs. The NF-κB family includes p65 (RelA), RelB, c-Rel, p100/p52, and p105/p50 and exists as heterodimeric or homodimeric proteins, typically a heterodimer of p50 and RelA/p65 subunits. Under normal conditions, NF-κB exists in the cytoplasm in an inactive form associated with IκB inhibitory proteins. A variety of cellular stimuli such as cytokines, lipopolysaccharide (LPS), and phorbol 12-myristate 13-acetate (PMA) induce phosphorylation of IκB with a concomitant decrease in IκB levels through the ubiquitin–proteasome system [3]. IκB phosphorylation is regulated by the β subunit of mature IκB kinase (IKK) complex composed of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit: IKKγ/NEMO [4]. This pathway is designated as the canonical or classical pathway. After degradation of IκB, the released NF-κB translocates into the nucleus and activates its gene transcription. At a later time point, IκB levels were recovered because of the NF-κB transcriptional response of the IκB promoter, thereby terminating its transcriptional function [5]. In the non-canonical or alternative pathway, IKKα is activated. It phosphorylates p100, generating p52 and RelB heterodimers, which translocate to the nucleus.

Diacylglycerol kinase (DGK) is an enzyme that phosphorylates diacylglycerol (DG) to generate phosphatidic acid (PA) [6–9]. Actually, DG is well known to regulate various cellular functions through the DG-responsive C1 domain, which is included in signaling molecules such as protein kinase C (PKC) [10–13]. Because PA, the reaction product of DGK, has also been reported to regulate many signaling proteins such as Raf-1 and mTOR, DGK is probably a key regulator of these two signaling molecules [14]. Of the DGK family, DGKζ is characterized by its nuclear localization signal (NLS) and nuclear export signal (NES) [15–18], suggesting that this isozyme shuttles between the nucleus and the cytoplasm [19–24]. Recent reports have described that DGKζ is intimately involved in immune and inflammatory responses [25–28]. DGKζ-deficient T cells are hyper-responsive to T cell receptor stimulation [29]. Furthermore, DGKζ deficiency increases resistance to endotoxin shock but enhances susceptibility to *Toxoplasma gondii* infection [30]. It remains to be determined how DGKζ is involved in NF-κB pathway, which plays a critical role in inflammatory processes.

* Corresponding author at: Department of Anatomy & Cell Biology, Yamagata University School of Medicine, Iida-Nishi 2-2-2, Yamagata 990-9585, Japan. Tel.: +81 23 628 5207; fax: +81 23 628 5210.
E-mail address: kgoto@med.id.yamagata-u.ac.jp (K. Goto).

http://dx.doi.org/10.1016/j.bbamcr.2014.11.011
0167-4889/

This study examined the effects exerted by DGKζ on the regulatory mechanism of cytokine-induced NF-κB pathway. We demonstrate that, upon stimulation with TNF-α, DGKζ knockdown or deletion enhances IKKβ phosphorylation and IκB degradation, thereby leading to accelerated nuclear translocation and retention of the NF-κB p65 subunit. Additionally, we found that DGKζ deficiency promotes enhanced phosphorylation of p65 subunit and its association with CBP transcriptional coactivator. These conditions increase NF-κB transcriptional activity. The present data suggest that DGKζ is a negative regulator of TNF-α-induced NF-κB pathway.

2. Materials and methods

2.1. Cell culture and reagents

Mouse embryonic fibroblasts (MEFs) obtained from wild-type (C57BL6) and DGKζ-KO mice were immortalized by transfection of SV40 plasmid DNA [29,31,32]. HeLa cells, MEFs, and A549 cells were grown at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Wako) with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin sulfate. Following 4 h serum starvation in DMEM containing 0.1% bovine serum albumin (BSA), cells were treated with either 20 ng/ml TNF-α (Miltenyi Biotec) or 50 ng/ml IL-1β (Miltenyi Biotec). In the control cells, 0.1% BSA vehicle was added to the medium. Anti-DGKζ antibody was produced as described previously [18,24]. Anti-NF-κB p65 (1:1000; #8242), anti-IκBα (1:1000; #4814), anti-IKKα (1:1000; #2682), anti-IKKβ (1:1000; #2370), anti-phospho-p65 Ser536 (1:1000; #3033), anti-phospho-p65 Ser468 (1:1000; #3039), anti-phospho-IκBα Ser32 (1:1000; #2850), anti-phospho-IKKα/β Ser176/180 (1:1000; #2697), anti-phospho-Akt Ser473 (1:1000; #4060), anti-phosho-p38 MAPK Thr180/Tyr182 (1:1000; #4511), anti-p38 MAPK (1:1000; #9212), anti-phospho-ERK Thr202/Tyr204 (1:1000; #4370), anti-ERK (1:1000; #4695), anti-phospho-JNK Thr183/Tyr185 (1:1000; #4668), and anti-JNK (1:1000; #9252) antibodies were purchased from Cell Signaling Technology. Anti-Rho GDIα (0.1 μg/ml, sc-360) and anti-Lamin B (0.2 μg/ml, sc-6216) antibodies were from Santa Cruz Biotechnology. Anti-β-actin (1:5000; AC-15) antibody was obtained from Sigma Aldrich.

2.2. RNA silencing

Human specific DGKζ siRNAs (siDGKζ7: 5′-AUUGAGAUACCAGAGG AAAGACUGG-3′, siDGKζ8: 5′-UAGAACAUCUUAUUCCGAAAGCGGC-3′) were obtained from Life Technologies. Control siRNA (All stars negative control) was purchased from Qiagen. The siRNAs were transfected using RNAiMAX (Life Technologies).

2.3. Immunostaining

HeLa cells and MEFs were seeded onto coverslips in a 35-mm dish for the experiments. The cells were fixed with 4% paraformaldehyde in PBS at room temperature (RT) for 15 min, permeabilized with 0.1% Triton X-100/PBS for 15 min, and blocked with 5% normal goat serum (NGS) for 20 min [33]. After washing with PBS three times, the cells were incubated with rabbit anti-p65 NF-κB antibody followed by secondary antibody conjugated with Alexa 488 (Life Technologies). Immunofluorescence images were obtained with a confocal laser microscope (LSM510, Carl Zeiss).

2.4. Immunoblotting

Cells were washed with ice-cold PBS and then lysed in lysis buffer composed of 100 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40, 1 mM Na_3VO_4, 50 mM NaF, 1 mM β-glycerophosphate, protease inhibitor cocktail (Sigma Aldrich). After centrifugation for 10 min at 15,000 ×*g* at 4 °C, the supernatant was transferred into a new tube and determined for the protein concentration using Pierce BCA Protein Assay Reagent (Thermo Fisher Scientific). In some experiments, cell lysates were separated into cytosolic and nuclear fractions using the ProteoExtract subcellular proteome extraction kit (Millipore) according to the manufacturer's instruction. Samples were resolved by SDS-PAGE and transferred to a PVDF membrane (Immobilon-P, Millipore). The membranes were incubated with primary antibodies, and the immunoactive proteins were visualized by using the Immobilon Western Chemiluminescent HRP Substrate (Millipore). Band intensities were quantified by densitometry using image J (National Institutes of Health, Bethesda) as described [34].

2.5. Luciferase reporter assay

HeLa cells were transfected with DGKζ siRNA (siDGKζ) or control siRNA (siControl). After 24 h, the cells were then transfected with pNF-κB-Luc (2 μg, Clontech) and pRL null (0.04 μg, TOYO INK) using Lipofectamine 2000 (Life Technologies) for further 24 h. Cells were stimulated with TNF-α (20 ng/ml) for 8 h and lysed in PicaGene Dual Cell Culture Lysis Reagent (TOYO INK) for 15 min at 4 °C. Cell extracts were collected and cleared by centrifugation at 15,000 ×*g*, and protein concentration was determined with the Pierce BCA Protein Assay Reagent. Renilla and firefly luciferase activities were measured using a Luminescenncer-Octa (ATTO).

2.6. RT-PCR

Total RNA was extracted from wild-type (WT) and DGKζ-knockout (KO) MEFs using TRIzol reagent (Life Technologies). First strand cDNA was synthesized from 2 μg of total RNA using reverse transcriptase (PrimeScript 1st strand cDNA Synthesis Kit, Clontech) according to the manufacturer's instruction. Polymerase chain reaction amplification was performed with Quick Taq HS DyeMix (Toyobo) using gene specific oligonucleotide primers: mouse DGKζ: 5′-TTACACAGATGAGCCTGT GTC-3′ (sense), 5′-GTTTCAGGTCCTGAATCTTGC-3′ (antisense); TNF-α: 5′-TAGCCCACGTCGTAGCAAAC-3′ (sense), 5′-AAGTAGACCTGCCCGGAC TC-3′ (antisense); β-actin: 5′-CCCATGCCATCCTGCGTCTG-3′ (sense); 5′-CGTCATACTCCTGCTTGCTG-3′ (antisense). The PCR condition was one cycle of pre-denaturation at 94 °C for 2 min followed by 25–28 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and elongation at 68 °C for 1 min, and then at 68 °C for 10 min. PCR products were separated by 2% agarose gels, stained with ethidium bromide, and visualized by Printgraph (ATTO).

2.7. Immunoprecipitation

Wild-type (WT) and DGKζ-KO MEFs were treated with or without TNF-α (20 ng/ml) for 10 min. Cells were lysed in lysis buffer (20 mM Tris–HCl, pH 7.4, 50 mM NaCl, 1 mM Na_3VO_4, 50 mM NaF, 1% Triton X-100, and protease inhibitor cocktail). After centrifugation at 16,000 ×*g* for 10 min 4 °C, the supernatant was transferred to a new tube and mixed with protein A-Sepharose beads (GE Healthcare) for 1 h at 4 °C to eliminate non-specific binding. Precleared supernatant (1 mg) was mixed with 1 μg of rabbit anti-p65 NF-κB antibody and incubated with protein A-Sepharose beads for 1 h. After washing with lysis buffer four times, the immunocomplex was boiled for 10 min in SDS sample buffer (New England Biolabs). The sample was applied to an SDS-PAGE gel and subjected to immunoblot analysis.

3. Results

3.1. Subcellular dynamics of NF-κB p65 subunit under conditions of DGKζ downregulation

Under resting conditions, NF-κB, consisting typically a heterodimer of p65/RelA and p50 subunits, is sequestered in the cytoplasm in

association with IκB proteins that mask a nuclear localization signal (NLS) of the p65 subunit. Consequently, nuclear translocation of NF-κB is a critical regulatory step for transcriptional activity upon stimulation [35]. Previous reports describe that following cellular exposure to TNF-α, p65 subunit oscillates dynamically between the nucleus and the cytoplasm in an approximately 1 h cycle [36]. Therefore we examined whether downregulation of DGKζ might affect the dynamics of NF-κB p65 subcellular localization.

First, HeLa cells transfected with control (siControl) or DGKζ siRNA (siDGKζ) were subjected to the time course analysis of p65 subunit immunocytochemistry following TNF-α stimulation (Fig. 1). Under resting conditions, p65 staining was detected primarily in the cytoplasm in both control and DGKζ knockdown cells. In the control cells, p65 subunit was partially observed in the nucleus at 15 min of stimulation and became dominant to the nucleus, peaking at 30 min. Thereafter, at 60 min, the p65 subunit was evenly distributed in the nucleus and cytoplasm or rather dominantly in the cytoplasm. However, in DGKζ knockdown cells, TNF-α stimulation accelerated the nuclear translocation of p65 subunit: p65 staining was observed predominantly in the nucleus at 15 and 30 min, and nuclear dominant staining of p65 remained in approximately 50% of cells at 60 min.

We performed the same experiment in DGKζ-deficient MEFs. As presented in Fig. 2, in DGKζ-null MEFs, the p65 subunit was translocated promptly to the nucleus, where it was retained for a longer time than wild-type cells, which is more clearly observed compared with DGKζ knockdown cells. We also stimulated cells with IL-1β, which produced similar results (Supplementary Fig. 1).

We next complemented these morphological experiments by biochemical analyses. After 15 min of TNF-α stimulation, the time point of maximum p65 nuclear translocation, cell lysates were fractionated into cytosolic (cyt) and nuclear (nuc) fractions, which were subjected to immunoblot analysis with p65 antibody. Similar to the immunohistochemical data, p65-immunoreactive band in the nuclear fraction was more intense in DGKζ-KO MEFs (Fig. 2C, lane 4) compared with that in wild-type MEFs (lane 2). Collectively, these results suggest that DGKζ downregulation promotes nuclear translocation of the NF-κB p65 subunit and prolongs its nuclear retention.

3.2. DGKζ downregulation facilitates IκB degradation

Recently, two distinct NF-κB activation pathways have been identified, i.e., the classical and alternative NF-κB signaling pathways [2]. Of

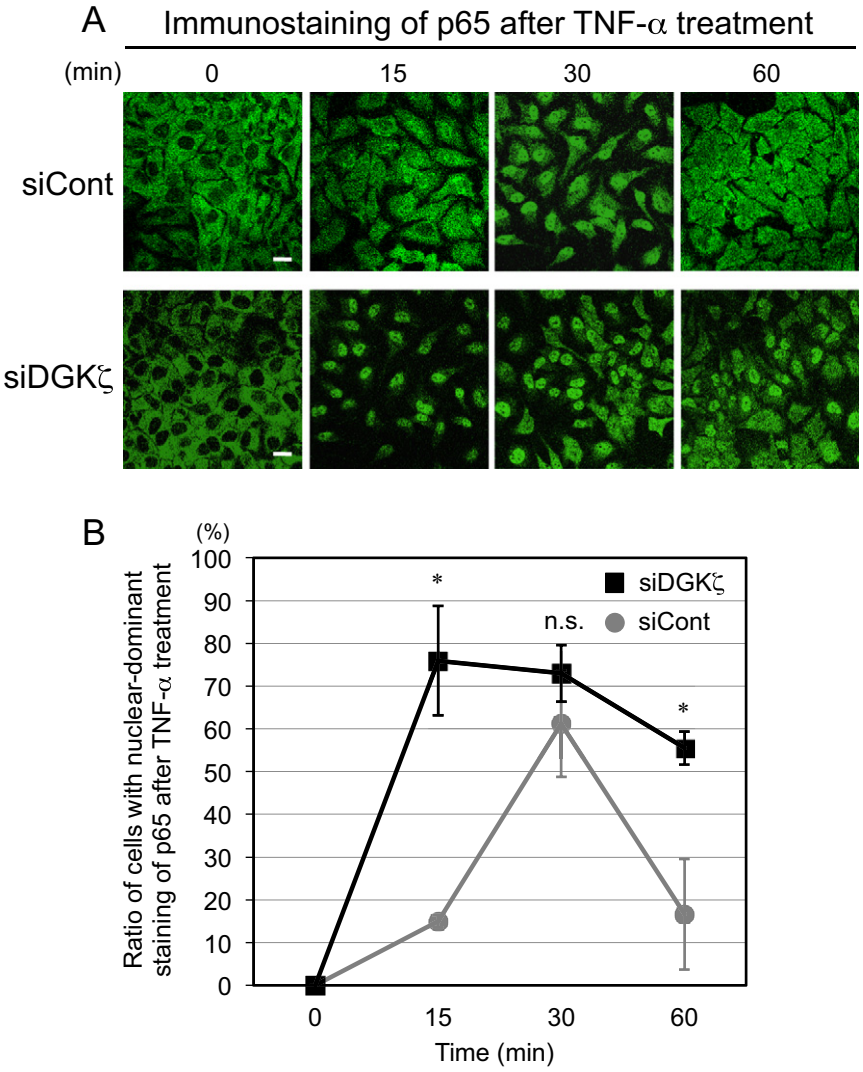

Fig. 1. Effect of RNA silencing of DGKζ on NF-κB p65 subcellular dynamics following TNF-α stimulation. (A) HeLa cells were transfected with nontargeted control siRNA (siCont) or DGKζ siRNA (si DGKζ). After 48 h, cells were stimulated with TNF-α (20 ng/ml). Cells were fixed with 4% paraformaldehyde at the time points indicated and immunostained with anti-p65 NF-κB antibody. Scale bars, 10 μm. (B) Fifty cells were counted for the subcellular localization of p65 subunit in (A) and classified into nuclear-dominant (nucleus > cytoplasm) or cytoplasmic-dominant (nucleus ≤ cytoplasm). The presented data are mean ± SD of three separate experiments. Asterisks indicate significance (Welch two sample *t*-test), $^{*}P < 0.05$; n.s., not statistically significant.

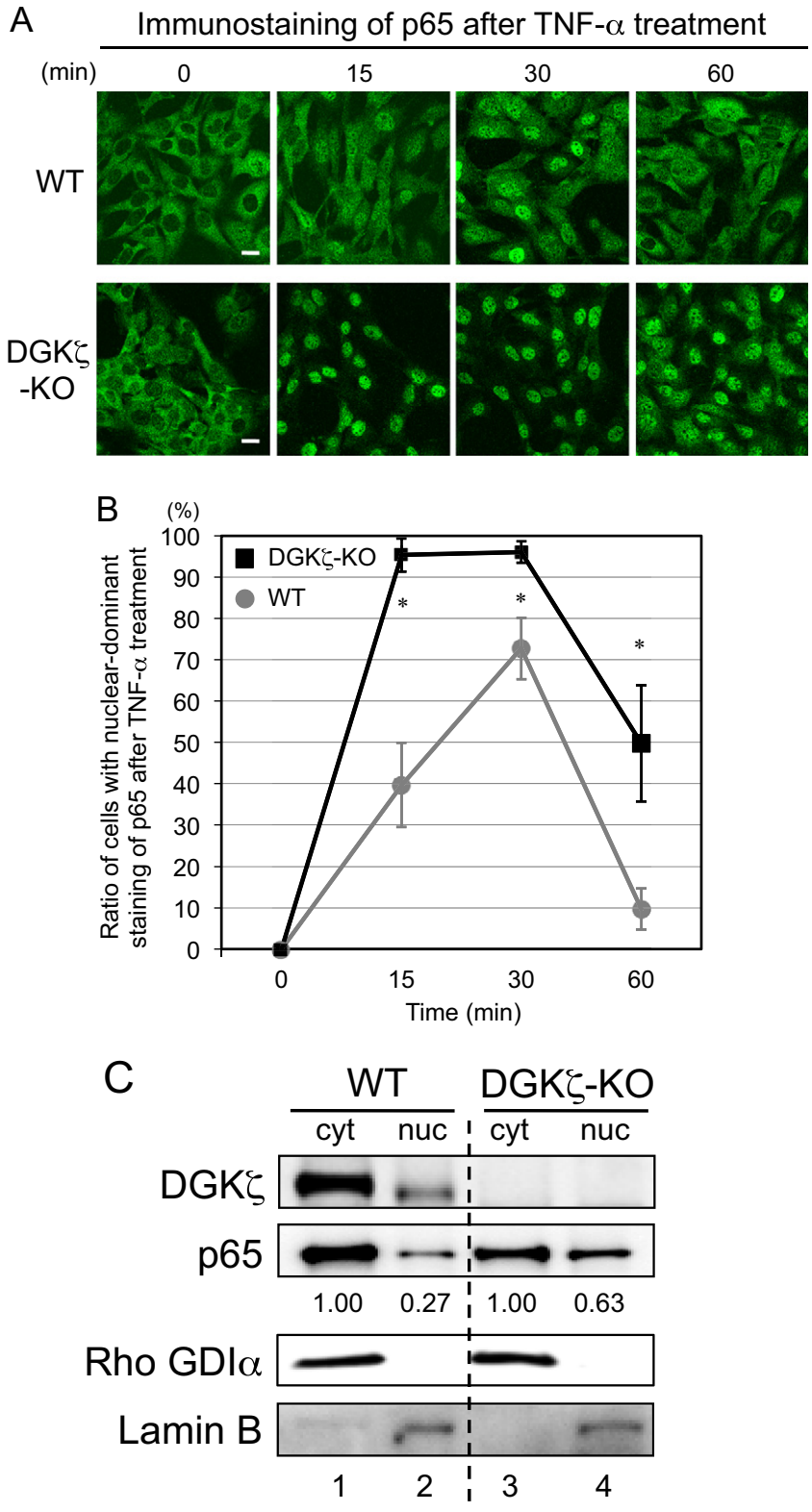

Fig. 2. NF-κB p65 subcellular dynamics in DGKζ-deficient MEFs following TNF-α stimulation. (A) Subconfluent wild-type (WT) or DGKζ-deficient (DGKζ-KO) MEFs were stimulated with TNF-α (20 ng/ml) for indicated time and immunostained with anti-p65 NF-κB antibody. Scale bars, 10 μm. (B) Fifty cells were counted for the subcellular localization of p65 subunit in (A) and classified into nuclear-dominant (nucleus > cytoplasm) or cytoplasmic-dominant (nucleus ≤ cytoplasm). The presented data are mean ± SD of three separate experiments. Asterisks indicate significance (Welch two sample *t*-test), $^{*}P < 0.05$; n.s., not statistically significant. (C) Biochemical distribution of p65 subunit in WT and DGKζ-KO MEFs. After 15 min of TNF-α stimulation, cell lysates were fractionated into cytosolic (cyt) and nuclear (nuc) fractions, as described in Section 2. Equal volumes of fractions were subjected to immunoblot analysis using anti-p65, anti-Rho GDIα (cytosolic marker), and anti-Lamin B (nuclear marker). Immunoreactive bands of IκBα were quantified by densitometry and normalized to the value of sample in cytosolic fraction of each MEF. A representative result of at least three repeated experiments is shown.

the two pathways, classical NF-κB activation is regulated by inducible IκB degradation. This pathway can be activated rapidly and transiently by various stimuli such as mitogens and cytokines. IκB degradation engenders unmasking of the NLS of NF-κB p65 subunit, thereby facilitating its nuclear translocation. Because NF-κB nuclear translocation is reported to be closely associated with IκB degradation, we examined the time course changes in the levels of IκB in control and DGKζ knockdown cells after stimulation with TNF-α (Fig. 3A). In control cells, IκB levels were decreased to about 40–50% of resting levels at 15–30 min of TNF-α stimulation and returned to 70% at 60 min. However, in DGKζ knockdown cells, IκB levels were massively attenuated to less than 10% of resting levels and were recovered to at most 30% at 60 min. Similar results were obtained for the experiment using another cell line, A549 cells derived from human alveolar adenocarcinoma (Supplementary Fig. 4). Furthermore, we confirmed these findings in the experiments using DGKζ-deficient MEFs with stimulation of TNF-α (Fig. 3B) and IL-1β (Supplementary Fig. 2).

We next performed rescue (add-back) experiments using rat DGKζ, which is resistant to siDGKζ for human sequence (Supplementary Fig. 5). Reintroduction of wild-type rat DGKζ in siDGKζ-treated HeLa cells reverted IκB downregulation after TNF-α stimulation (lane 6 versus lane 4) to the extent of the control level (lane 2). It should be noted that reintroduction of catalytically inactive rat DGKζ (rDGKζ-KD) in siDGKζ-treated HeLa cells also reverted IκB expression after TNF-α stimulation to the extent of wild-type rDGKζ and the control levels (Supplementary Fig. 6, lane 6 versus lanes 4 and 2). Taken together, the results suggest that DGKζ downregulation facilitates IκB degradation following cytokine stimulation and that DGKζ exerts a regulatory effect on IκB levels independently of its kinase activity.

3.3. Phosphorylation of NF-κB regulatory components is facilitated by DGKζ downregulation

Phosphorylation targets IκB for ubiquitination followed by proteasome-mediated degradation [3,37,38]. IκB kinase (IKK) is the protein kinase complex responsible for IκB phosphorylation in response to proinflammatory stimuli [39]. IKK is a multisubunit complex that includes two catalytic subunits, IKKα and IKKβ. An earlier study showed that IKKβ is responsible for the proinflammatory cytokine-induced activation of NF-κB [40]. The mechanisms that activate the IKK complex are not understood completely, but activation is known to require phosphorylation of the IKKβ activation loop [41].

Therefore we next investigated the phosphorylation status of IKK, together with NF-κB p65 subunit, in DGKζ-deficient MEFs during TNF-α stimulation (Fig. 4). Because IκB degradation was observed in the early phase of stimulation (15–30 min), as depicted in Fig. 3, we specifically examined the time points within the first 15 min. IκB attenuation was observed at 10 min of stimulation in wild-type MEFs, although it became apparent at 5 min in DGKζ-deficient MEFs. Consistent with this time course, phosphorylation of IKKβ (upper band) was increased at 5–10 min in DGKζ-deficient MEFs, although IKKα phosphorylation (lower band) showed no marked changes between wild-type and DGKζ-deficient MEFs. Furthermore, the results show that p65 subunit phosphorylation at Ser536 was upregulated at 5–10 min in DGKζ-deficient MEFs; so was phosphorylation at Ser468 shortly later at 10–15 min. These results suggest that DGKζ deletion activates the phosphorylation reaction of the regulatory components of NF-κB pathway.

NF-κB transactivation is reportedly mediated by the activation of mitogen-activated protein kinase (MAPK), PI3K-Akt and PKC pathways [2,39]. Therefore we next examined several of those upstream kinases responsible for increased phosphorylation of IKKβ and p65 subunit after TNF-α stimulation. We used the same experimental design as that shown in Fig. 4, which enables us to examine the upstream kinases using p38 MAPK, ERK, and Akt antibodies. As presented in Fig. 5, phosphorylation levels of p38 MAPK and ERK were apparently increased slightly at 10–15 min in DGKζ-deficient MEFs compared with the wild-type one. Akt phosphorylation showed no apparent differences between the two groups (data not shown).

Regarding involvement of PKC, thrombin induces PKC-δ activation in endothelial cells. This event is necessary for IKKβ and p38 MAPK activation [42]. Furthermore, PKC-δ-dependent activation of IKKβ and p38 MAP kinase contributes to the mechanism of thrombin-induced ICAM-1 expression by activating NF-κB in the cytoplasm and increasing the transactivation potential of NF-κB p65 subunit in the nucleus. Therefore,

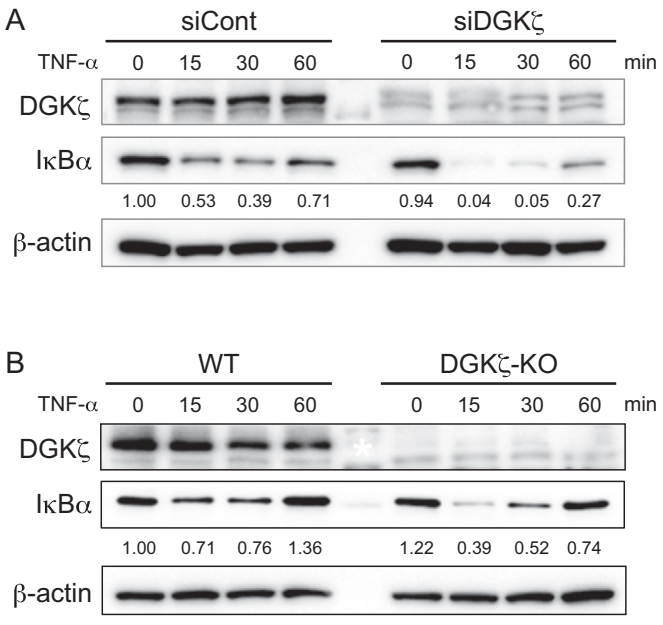

Fig. 3. Effect of DGKζ downregulation on IκB expression after TNF-α stimulation. HeLa cells transfected with control siRNA (siCont) or DGKζ siRNA (si DGKζ) for 48 h (A) and MEFs from wild-type (WT) or DGKζ-deficient (DGKζ-KO) mice (B) were stimulated with TNF-α (20 ng/ml). Cells were collected at the time points indicated and the lysates were subjected to immunoblot analysis (20 μg protein/lane) using anti-IκBα antibody. Anti-β-actin antibody was used as a control. Immunoreactive bands of IκBα were quantified by densitometry and normalized to the value of sample at 0 min obtained in siCont-treated or WT MEFs. A representative result of at least three repeated experiments is shown.

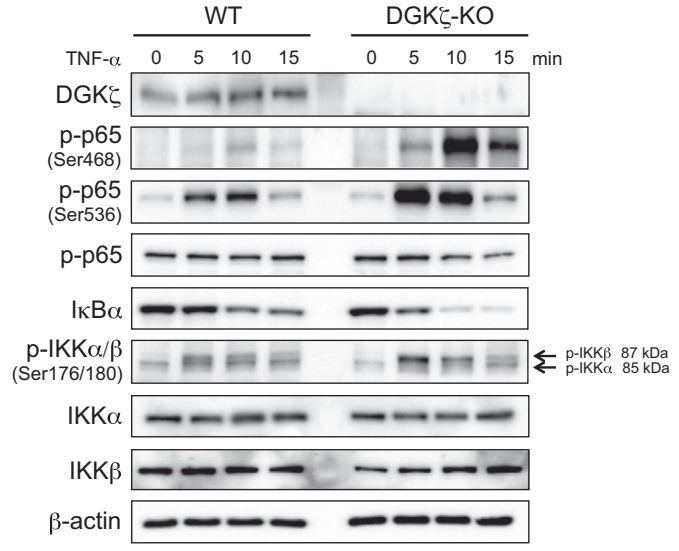

Fig. 4. Effect of DGKζ deficiency on the phosphorylation status of p65 subunit and IKK after TNF-α stimulation. Subconfluent wild-type (WT) or DGKζ-deficient (DGKζ-KO) MEFs were stimulated with TNF-α (20 ng/ml). Cells were collected at the time points indicated and subjected to immunoblot analysis (20 μg protein/lane) using anti-phospho-p65 (Ser468), anti-phospho-p65 (Ser536), anti-p65 (total), anti-IκBα, anti-IKKα, anti-IKKβ, and anti-phospho-IKKα/β (Ser176/180), antibodies. Bands for phospho-IKKβ and -IKKα were indicated by arrows. Anti-β-actin antibody was used as a control.

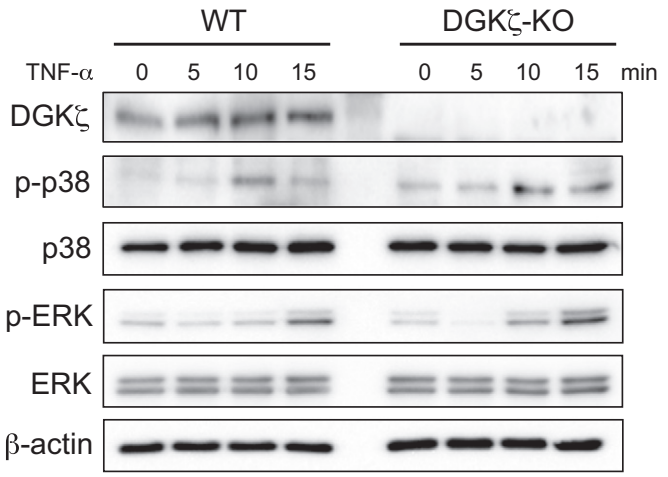

Fig. 5. Effect of DGKζ deficiency on the phosphorylation status of upstream kinases after TNF-α stimulation. Samples obtained in Fig. 4 were subjected to immunoblot analysis using anti-p38 MAPK, anti-phosho-p38 MAPK Thr180/Tyr182, anti-ERK, and anti-phospho-ERK Thr202/Tyr204 antibodies. Anti-β-actin antibody was used as a control.

we performed experiments using pan-PKC inhibitor on the phosphorylation of IκB and p65, together with IκB degradation, in DGKζ-deficient MEFs. No effect on these parameters was apparent in the presence or absence of PKC inhibitor (Supplementary Fig. 3).

3.4. DGKζ knockdown enhances the association of p65 subunit with CBP and NF-κB transactivation activity

Having shown that DGKζ downregulation/deletion promotes NF-kB p65 subunit nuclear translocation, prolongs its nuclear retention, and upregulates p65 phosphorylation, we next asked whether these conditions modulate the association of p65 subunit with CBP/p300 coactivator and subsequent NF-κB transactivation activity. An earlier study showed that p65 phosphorylation at Ser536 and/or Ser276 promotes enhanced interaction of p65 subunit and CBP/p300 and the following NF-κB transcriptional activation [43]. Therefore, we first performed the coimmunoprecipitation experiments with DGKζ-deficient MEFs (Fig. 6). The results showed that DGKζ deletion enhances the association of p65 subunit with CBP significantly after 10 min stimulation with TNF-α compared with wild-type MEFs. It is noteworthy that this

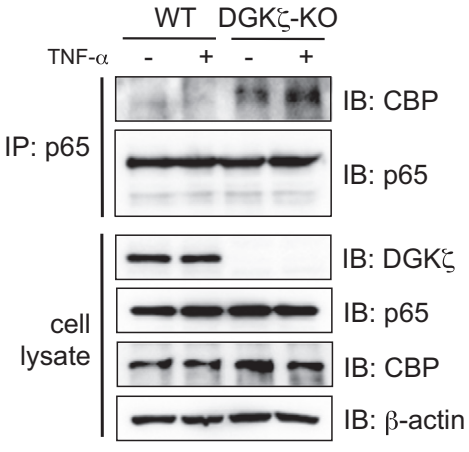

Fig. 6. Effect of DGKζ deficiency on the association of p65 subunit and transcriptional coactivator CBP. Subconfluent wild-type (WT) or DGKζ-deficient (DGKζ-KO) MEFs were treated with or without TNF-α (20 ng/ml) for 10 min. The lysates were mixed with anti-p65 antibody combined with protein-G-Sepharose beads and incubated for 6 h at 4 °C. Cell lysates and the immunoprecipitates were analyzed by immunoblotting using anti-CBP and anti-p65 antibodies. Anti-β-actin antibody was used as a control. IP, immunoprecipitation; IB, immunoblot.

association was also evident in the absence of TNF-α stimulation. These findings suggest that p65 subunit closely associates with CBP in the absence or presence of stimulation. We next performed the NF-κB luciferase reporter assay using DGKζ knockdown cells (Fig. 7). HeLa cells were transfected with either siControl or siDGKζ along with NF-κB reporter and internal control pRL. In the control cells, NF-κB transcriptional activity was increased approximately 5-fold after TNF-α stimulation. In contrast, in DGKζ knockdown cells, the activity was increased 8–12-fold after stimulation. Additionally, RT-PCR analysis showed prominent upregulation of NF-κB target gene TNF-α mRNA levels in DGKζ-null MEFs compared with the wild-type one (Fig. 8).

4. Discussion

The results of this study demonstrated that DGKζ downregulation engenders enhancement of NF-κB pathway in response to cytokines such as TNF-α and IL-1β. In response to these cytokines, the NF-κB p65 subunit is accelerated for its nuclear translocation, which represents the major regulatory step for the activation, under DGKζ-knockdown or DGKζ-deficient conditions. In addition, DGKζ deficiency promotes enhanced interaction of p65 subunit with CBP coactivator. The resulting prolonged retention of p65 subunit in the nucleus, together with its enhanced association with transcriptional coactivator, might engender augmented NF-κB transactivation activity upon cytokine stimulation.

NF-κB has often been termed a 'central mediator of the immune response' because it regulates the expression of inflammatory cytokines, chemokines, immunoreceptors, and cell adhesion molecules [44]. This fact indicates that NF-κB induces the expression of inflammatory cytokines and chemokines, and in turn, is induced by them, suggesting a

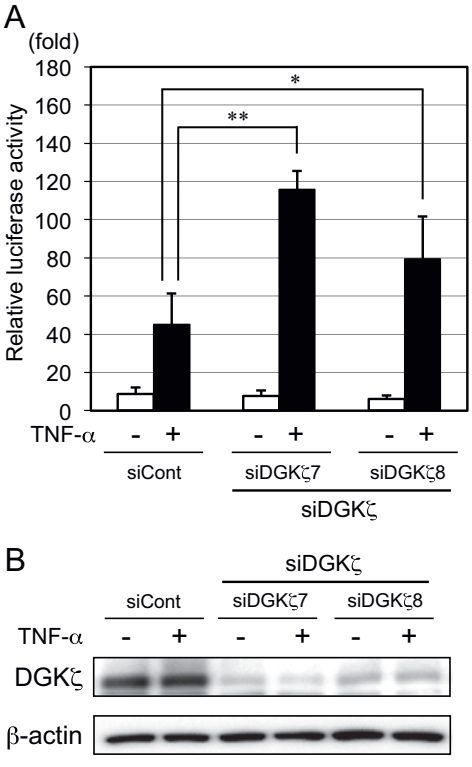

Fig. 7. NF-κB luciferase reporter assay. HeLa cells were co-transfected with control siRNA (siCont) or DGKζ siRNA (siDGKζ7 or siDGKζ8) along with pNF-κB-Luc (2 μg) and internal control pRL null (0.04 μg) reporter constructs. After 48 h, cells were treated with or without TNF-α (20 ng/ml) for 8 h, and Renilla and firefly luciferase activities were measured (A). The presented data are mean ± SD of four separate experiments. Asterisks indicate significance (ANOVA followed by Dunnet), $^{**}P < 0.01$; $^{*}P < 0.05$. (B) Cell lysates (20 μg protein/lane) were subjected to immunoblot analysis to confirm knockdown of DGKζ protein expression. Anti-β-actin antibody was used as a control.

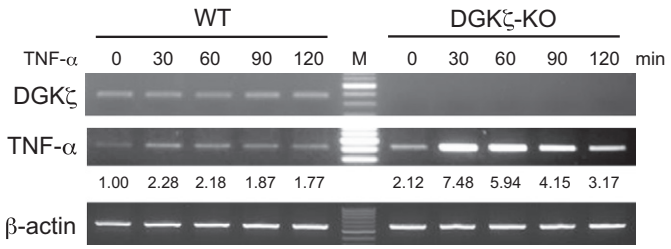

Fig. 8. Expression of mRNA for NF-κB target gene TNF-α in DGKζ-deficient MEFs after TNF-α stimulation. Subconfluent wild-type (WT) or DGKζ-deficient (DGKζ-KO) MEFs were stimulated with TNF-α (20 ng/ml). Cells were collected at the time points indicated and subjected to PCR analysis using specific primer for TNF-α. PCR was performed for 25–28 cycles. β-Actin primer was used as a control. PCR bands were quantified by densitometry and normalized to the value of sample at 0 min obtained in WT MEFs.

positive feedback mechanism for NF-κB. In this regard, the present findings demonstrate clearly that DGKζ knockdown or deletion accelerates this positive feedback mechanism for NF-κB. These conditions might provide the basis for chronic or excessive inflammatory conditions such as rheumatoid arthritis, asthma, and inflammatory bowel disease [45].

Of the DGK family, previous studies have shown that DGKα and DGKζ are abundantly expressed in the immune system [15,46]. Both of these isozymes are proposed to exert a suppressive effect on T cell receptor (TCR) response [47,48]. In T cells, DGKα and DGKζ inhibit DG-mediated signaling following TCR engagement and prevent T-cell hyperactivation. Consequently, DGKα and DGKζ synergistically promote T-cell anergy and are critical for T-cell tolerance [25]. Suppressive effects of DGKζ on T cells are revealed by the phenotype of DGKζ-deficient mice, which show Ras/MAPK activation, together with enhanced expression of the CD69 and CD25 activation markers [29].

In dendritic cells and macrophages, however, DGKζ positively regulates Toll-like Receptor (TLR)-induced proinflammatory cytokine production through its product PA [30]. Earlier studies have shown that both DG and PA are produced in macrophages following LPS (a TLR4 ligand) and lipopeptide (a TLR2 ligand) stimulation [49–51]. TLR-mediated responses are crucially important for host defense against microbial organisms such as *T. gondii*. Host defense against this organism is initiated by dendritic cells, macrophages, and other antigen-presenting cells after recognition of pathogen-associated molecular patterns through TLR2 and TLR11. Deficiency of these TLRs increases host susceptibility to *T. gondii* infection [52,53]. TLR signaling and the subsequent production of IL-12 by immune cells induce Th1 adaptive immune responses and IFN-γ production, which provides critical resistance to *T. gondii* infection [54]. In this regard, DGKζ deficiency reportedly results in impaired cytokine (IL-12 and TNF-α) production following TLR stimulation, increased resistance to endotoxin shock, and enhanced susceptibility to *T. gondii* infection [30]. The increased susceptibility of DGKζ-deficient mice to *T. gondii* infection is apparently correlated with inhibition of Th1 immune responses in vivo [30]. Collectively, previous findings demonstrate that DGKζ exerts a suppressive effect on TCR through DG metabolism in T cells, although it exerts a positive effect on TLR-induced proinflammatory cytokine production through its product PA in dendritic cells and macrophages.

This study shows that DGKζ deficiency enhances TNF-α-induced NF-κB signaling pathway in HeLa cells. The remarkable diversity of stimuli is well known to engender activation of NF-κB, which might be mediated by signaling cascades that culminate in IKK activation, the final common pathway for the NF-κB activation. In this regard, we found that upon TNF-α challenge, DGKζ deficiency selectively activates the IKKβ subunit while leaving IKKα phosphorylation unchanged. This result suggests that DGKζ exerts a suppressive effect on the canonical NF-κB pathway. TLR signalings are mediated by the myeloid differentiation primary response protein 88 (MyD88)-dependent pathway, which is used by most TLRs except TLR3 [55]. This pathway is initiated after association of MyD88 with the TLRs during microbial recognition. MyD88 in turn recruits IL-1R-associated kinase 1 and 4 (IRAK1&4), TNFR-associated factor 6 (TRAF6), and other signaling molecules, leading to IKK complex activation. Considering previous and present findings together, they can be summarized as follows: DGKζ deficiency enhances TNF-α-induced NF-κB transactivation, but reduces TLR-mediated NF-κB pathway. These findings suggest that 1) DGKζ exerts positive and negative effects on the canonical pathway of NF-κB activation in a context-dependent manner; and 2) DGKζ acts on some upstream molecule(s) of IKK complex phosphorylation.

DGKα is also involved in the NF-κB signaling [56,57]. DGKα is shown to regulate TNF-α-dependent NF-κB activation positively in AKI melanoma cells, which contrasts sharply to the effect exerted by DGKζ. How do these DGK isozymes exert opposite effects on the NF-κB signaling pathway under the same stimulation? Clues to address this issue might be provided by their different regulatory mechanisms on the IκB and the phosphorylation events of p65 subunit. DGKα knockdown exerts no apparent effect on IκB protein levels, suggesting that DGKα does not influence subcellular dynamics of the p65 subunit. In contrast, DGKζ deficiency engenders facilitated degradation of IκB by IKKβ activation, thereby causing enhanced p65 nuclear translocation. With regard to the phosphorylation events, p65 subunit is well known to be subjected to phosphorylation at several sites, including Ser276 [58,59], Ser311 [60], Ser468 [61], Ser529 [62], and Ser536 [63]. Of those, phosphorylation sites at Ser276, Ser311, Ser529, Ser468, and Ser 536 might modulate the transcriptional activity. In this respect, DGKα depletion attenuates the phosphorylation levels of Ser311, but does not affect those at Ser468 and Ser536. In this case, decreased phosphorylation at Ser311 is apparently mediated by PKCζ that is activated directly by PA [60,64], which engenders downregulation of NF-κB transcriptional activity [57]. On the other hand, DGKζ deficiency causes elevated levels of the phosphorylation of p65 subunit at Ser468 and Ser536, which is reportedly mediated by IKK complex [39,63,65], thereby contributing to upregulation of NF-κB activity. Pan-PKC inhibitor has no apparent effect on IκB degradation and p65-Ser536 phosphorylation in DGKζ-deficient MEFs, suggesting that DGKζ has no regulatory effect on PKCs in this case (Supplementary Fig. 3). Furthermore, our add-back experiments reveal that catalytically inactive DGKζ-KD as well as wild-type DGKζ reverts IκB expression after TNF-α stimulation (Supplementary Fig. 6). This finding raises a possibility that DGKζ functions as a scaffold protein to regulate IκB degradation, although detailed analysis remains to be done as to whether catalytically inactive DGKζ-KD reverts the phenotype on phosphorylation events of p65 subunit and IKKβ, together with CBP association and NF-κB transactivation activity. These results suggest that, under inflammatory conditions, DGKα and DGKζ act on distinct molecules involved in NF-κB pathway, respectively producing positive and negative regulation.

Another finding of this work is that DGKζ deficiency enhances the association of p65 subunit with the transcriptional coactivator CBP, which might contribute to accelerated transactivation activity. This association is shown to be promoted by phosphorylation of p65 at Ser536 and/or Ser276 [43]. In this regard, we find increased phosphorylation at Ser536 after TNF-α stimulation in DGKζ-deficient MEFs. However, DGKζ deficiency enhances the association of p65 subunit with CBP, even under basal conditions in the absence of TNF-α stimulation. How can this phenotype be explained? One possibility is that DGKζ deficiency might modulate phosphorylation of p65 subunit at Ser276, which is mediated by either protein kinase A (PKA) or mitogen-activated and stress-activated protein kinase-1 (MSK1) [59,66].

The other possibility involves previous observations that NF-κB p65 subunit and tumor suppressor p53 compete for limiting quantities of complexes containing the CBP/p300 coactivator proteins [67,68]. Both NF-κB and p53 are activated in response to similar stimuli, such as DNA damage and TNF-α [4]. p53 is generally a proapoptotic transcriptional factor whereas NF-κB promotes resistance to programmed cell death under most circumstances [69,70]. It is therefore suggested that

competition for CBP is a crucially important determinant of whether a cell proliferates or undergoes apoptosis [71]. In this regard, we reported previously that DGKζ deletion upregulates 53 protein levels under basal and DNA-damage conditions but simultaneously suppresses p53 transcriptional activity [72,73]. Together with the present findings, DGKζ deficiency augments transcriptional activity of NF-κB but attenuates that of p53. These features of DGKζ-deficient conditions might be explained by the hypothesis that DGKζ plays a role in sequestration of the limiting pool of CBP/p300 between p65 subunit and p53 and that DGKζ deficiency shifts CBP/p300 toward NF-κB p65 subunit to regulate the reciprocally antagonistic phenotypes of these transcription factors in an as yet unknown mechanism. However, this is not the whole explanation for the cross talk that happens between them because many other inducible transcription factors have been reported that also interact with CBP/p300 [74–76]. Further investigation must be undertaken to assess how DGKζ regulates sequestration of CBP/p300 transcriptional coactivators among various transcription factors to integrate the diverse functions of all these proteins.

Uncertainty remains in relation to the activation process of IKKβ under DGKζ-deficient conditions. Reportedly, the IKK complex is phosphorylated by upstream kinases such as MEKK1 [77], MEKK3 [78], and TGF-β-activated kinase 1 (TAK1) [79], NF-κB inducible kinase (NIK) [77], and Akt [80]. In this study, DGKζ-KO MEFs show slightly increased phosphorylation levels on p38 and ERK, although it remains undetermined whether such level of upregulation is sufficient to activate IKKβ or not. It also remains to be elucidated how the acetylation status for p65 subunit and histone proteins is modulated by enhanced association with CBP coactivator. Further studies are necessary to elucidate these issues.

5. Conclusion

This study revealed that DGKζ downregulation engenders facilitation of nuclear transport of p65 subunit and its interaction with CBP transcriptional coactivator upon cytokine stimulation, thereby enhancing NF-κB transactivation activity. These conditions might produce an augmented positive feedback mechanism of cytokine-induced NF-κB activity, which might cause aberrant NF-κB activation. Investigations must be conducted to ascertain detailed molecular mechanism of DGKζ on NF-κB pathway and how these NF-κB dynamics under DGKζ downregulation engender disorganized responses at the organism level.

Acknowledgements

This work was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (K.G.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.11.011.

References

[1] L. Verstrepen, T. Bekaert, T.L. Chau, J. Tavernier, A. Chariot, R. Beyaert, TLR-4, IL-1R and TNF-R signaling to NF-kappaB: variations on a common theme, Cell. Mol. Life Sci. 65 (2008) 2964–2978.
[2] J.A. DiDonato, F. Mercurio, M. Karin, NF-kappaB and the link between inflammation and cancer, Immunol. Rev. 246 (2012) 379–400.
[3] L. Baldi, K. Brown, G. Franzoso, U. Siebenlist, Critical role for lysines 21 and 22 in signal-induced, ubiquitin-mediated proteolysis of I kappa B-alpha, J. Biol. Chem. 271 (1996) 376–379.
[4] M.S. Hayden, S. Ghosh, Shared principles in NF-kappaB signaling, Cell 132 (2008) 344–362.
[5] S.C. Sun, P.A. Ganchi, D.W. Ballard, W.C. Greene, NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway, Science 259 (1993) 1912–1915.
[6] H. Kanoh, K. Yamada, F. Sakane, Diacylglycerol kinase: a key modulator of signal transduction? Trends Biochem. Sci. 15 (1990) 47–50.
[7] F. Sakane, S. Imai, M. Kai, S. Yasuda, H. Kanoh, Diacylglycerol kinases: why so many of them? Biochim. Biophys. Acta 1771 (2007) 793–806.
[8] K. Goto, Y. Hozumi, T. Nakano, S.S. Saino, H. Kondo, Cell biology and pathophysiology of the diacylglycerol kinase family: morphological aspects in tissues and organs, Int. Rev. Cytol. 264 (2007) 25–63.
[9] M.K. Topham, R.M. Epand, Mammalian diacylglycerol kinases: molecular interactions and biological functions of selected isoforms, Biochim. Biophys. Acta 1790 (2009) 416–424.
[10] Y. Nishizuka, Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C, Science 258 (1992) 607–614.
[11] N. Divecha, R.F. Irvine, Phospholipid signaling, Cell 80 (1995) 269–278.
[12] M.N. Hodgkin, T.R. Pettitt, A. Martin, R.H. Michell, A.J. Pemberton, M.J. Wakelam, Diacylglycerols and phosphatidates: which molecular species are intracellular messengers? Trends Biochem. Sci. 23 (1998) 200–204.
[13] M.J. Wakelam, Diacylglycerol—when is it an intracellular messenger? Biochim. Biophys. Acta 1436 (1998) 117–126.
[14] I. Merida, A. Avila-Flores, E. Merino, Diacylglycerol kinases: at the hub of cell signalling, Biochem. J. 409 (2008) 1–18.
[15] K. Goto, H. Kondo, A 104-kDa diacylglycerol kinase containing ankyrin-like repeats localizes in the cell nucleus, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 11196–11201.
[16] M. Bunting, W. Tang, G.A. Zimmerman, T.M. McIntyre, S.M. Prescott, Molecular cloning and characterization of a novel human diacylglycerol kinase zeta, J. Biol. Chem. 271 (1996) 10230–10236.
[17] C. Evangelisti, G.C. Gaboardi, A.M. Billi, A. Ognibene, K. Goto, P.L. Tazzari, J.A. McCubrey, A.M. Martelli, Identification of a functional nuclear export sequence in diacylglycerol kinase-zeta, Cell Cycle 9 (2010) 384–388.
[18] Y. Hozumi, T. Ito, T. Nakano, T. Nakagawa, M. Aoyagi, H. Kondo, K. Goto, Nuclear localization of diacylglycerol kinase zeta in neurons, Eur. J. Neurosci. 18 (2003) 1448–1457.
[19] M.K. Topham, M. Bunting, G.A. Zimmerman, T.M. McIntyre, P.J. Blackshear, S.M. Prescott, Protein kinase C regulates the nuclear localization of diacylglycerol kinase-zeta, Nature 394 (1998) 697–700.
[20] H. Ali, T. Nakano, S. Saino-Saito, Y. Hozumi, Y. Katagiri, H. Kamii, S. Sato, T. Kayama, H. Kondo, K. Goto, Selective translocation of diacylglycerol kinase zeta in hippocampal neurons under transient forebrain ischemia, Neurosci. Lett. 372 (2004) 190–195.
[21] Y. Suzuki, Y. Yamazaki, Y. Hozumi, M. Okada, T. Tanaka, K. Iseki, N. Ohta, M. Aoyagi, S. Fujii, K. Goto, NMDA receptor-mediated Ca(2+) influx triggers nucleocytoplasmic translocation of diacylglycerol kinase zeta under oxygen–glucose deprivation conditions, an in vitro model of ischemia, in rat hippocampal slices, Histochem. Cell Biol. 137 (2012) 499–511.
[22] S. Saino-Saito, Y. Hozumi, K. Goto, Excitotoxicity by kainate-induced seizure causes diacylglycerol kinase zeta to shuttle from the nucleus to the cytoplasm in hippocampal neurons, Neurosci. Lett. 494 (2011) 185–189.
[23] M. Okada, Y. Hozumi, T. Tanaka, Y. Suzuki, M. Yanagida, Y. Araki, C. Evangelisti, H. Yagisawa, M.K. Topham, A.M. Martelli, K. Goto, DGKzeta is degraded through the cytoplasmic ubiquitin–proteasome system under excitotoxic conditions, which causes neuronal apoptosis because of aberrant cell cycle reentry, Cell. Signal. 24 (2012) 1573–1582.
[24] M. Okada, Y. Hozumi, T. Ichimura, T. Tanaka, H. Hasegawa, M. Yamamoto, N. Takahashi, K. Iseki, H. Yagisawa, T. Shinkawa, T. Isobe, K. Goto, Interaction of nucleosome assembly proteins abolishes nuclear localization of DGKzeta by attenuating its association with importins, Exp. Cell Res. 317 (2011) 2853–2863.
[25] X.P. Zhong, R. Guo, H. Zhou, C. Liu, C.K. Wan, Diacylglycerol kinases in immune cell function and self-tolerance, Immunol. Rev. 224 (2008) 249–264.
[26] B.A. Olenchock, R. Guo, M.A. Silverman, J.N. Wu, J.H. Carpenter, G.A. Koretzky, X.P. Zhong, Impaired degranulation but enhanced cytokine production after Fc epsilonRI stimulation of diacylglycerol kinase zeta-deficient mast cells, J. Exp. Med. 203 (2006) 1471–1480.
[27] B.A. Olenchock, R. Guo, J.H. Carpenter, M. Jordan, M.K. Topham, G.A. Koretzky, X.P. Zhong, Disruption of diacylglycerol metabolism impairs the induction of T cell anergy, Nat. Immunol. 7 (2006) 1174–1181.
[28] M. Okada, Y. Hozumi, K. Iwazaki, K. Misaki, M. Yanagida, Y. Araki, T. Watanabe, H. Yagisawa, M.K. Topham, K. Kaibuchi, K. Goto, DGKzeta is involved in LPS-activated phagocytosis through IQGAP1/Rac1 pathway, Biochem. Biophys. Res. Commun. 420 (2012) 479–484.
[29] X.P. Zhong, E.A. Hainey, B.A. Olenchock, M.S. Jordan, J.S. Maltzman, K.E. Nichols, H. Shen, G.A. Koretzky, Enhanced T cell responses due to diacylglycerol kinase zeta deficiency, Nat. Immunol. 4 (2003) 882–890.
[30] C.H. Liu, F.S. Machado, R. Guo, K.E. Nichols, A.W. Burks, J.C. Aliberti, X.P. Zhong, Diacylglycerol kinase zeta regulates microbial recognition and host resistance to *Toxoplasma gondii*, J. Exp. Med. 204 (2007) 781–792.
[31] D.S. Regier, J. Higbee, K.M. Lund, F. Sakane, S.M. Prescott, M.K. Topham, Diacylglycerol kinase iota regulates Ras guanyl-releasing protein 3 and inhibits Rap1 signaling, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 7595–7600.
[32] H. Matsui, Y. Hozumi, T. Tanaka, M. Okada, T. Nakano, Y. Suzuki, K. Iseki, M.K. Topham, K. Goto, Role of the N-terminal hydrophobic residues of DGKepsilon in targeting the endoplasmic reticulum, Biochim. Biophys. Acta 1841 (2014) 1440–1450.
[33] H. Hasegawa, T. Nakano, Y. Hozumi, M. Takagi, T. Ogino, M. Okada, K. Iseki, H. Kondo, M. Watanabe, A.M. Martelli, K. Goto, Diacylglycerol kinase zeta is associated with chromatin, but dissociates from condensed chromatin during mitotic phase in NIH3T3 cells, J. Cell. Biochem. 105 (2008) 756–765.
[34] T. Nakano, Y. Hozumi, K. Iwazaki, K. Okumoto, K. Iseki, T. Saito, S. Kawata, I. Wakabayashi, K. Goto, Altered expression of diacylglycerol kinase isozymes in regenerating liver, J. Histochem. Cytochem. 60 (2012) 130–138.

[35] A.S. Baldwin Jr., The NF-kappa B and I kappa B proteins: new discoveries and insights, Annu. Rev. Immunol. 14 (1996) 649–683.
[36] D.E. Nelson, A.E. Ihekwaba, M. Elliott, J.R. Johnson, C.A. Gibney, B.E. Foreman, G. Nelson, V. See, C.A. Horton, D.G. Spiller, S.W. Edwards, H.P. McDowell, J.F. Unitt, E. Sullivan, R. Grimley, N. Benson, D. Broomhead, D.B. Kell, M.R. White, Oscillations in NF-kappaB signaling control the dynamics of gene expression, Science 306 (2004) 704–708.
[37] Z.J. Chen, L. Parent, T. Maniatis, Site-specific phosphorylation of IkappaBalpha by a novel ubiquitination-dependent protein kinase activity, Cell 84 (1996) 853–862.
[38] M. Roff, J. Thompson, M.S. Rodriguez, J.M. Jacque, F. Baleux, F. Arenzana-Seisdedos, R.T. Hay, Role of IkappaBalpha ubiquitination in signal-induced activation of NFkappaB in vivo, J. Biol. Chem. 271 (1996) 7844–7850.
[39] M.S. Hayden, S. Ghosh, Signaling to NF-kappaB, Genes Dev. 18 (2004) 2195–2224.
[40] M. Pasparakis, T. Luedde, M. Schmidt-Supprian, Dissection of the NF-kappaB signalling cascade in transgenic and knockout mice, Cell Death Differ. 13 (2006) 861–872.
[41] M. Delhase, M. Hayakawa, Y. Chen, M. Karin, Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation, Science 284 (1999) 309–313.
[42] A. Rahman, K.N. Anwar, S. Uddin, N. Xu, R.D. Ye, L.C. Platanias, A.B. Malik, Protein kinase C-delta regulates thrombin-induced ICAM-1 gene expression in endothelial cells via activation of p38 mitogen-activated protein kinase, Mol. Cell. Biol. 21 (2001) 5554–5565.
[43] L.F. Chen, S.A. Williams, Y. Mu, H. Nakano, J.M. Duerr, L. Buckbinder, W.C. Greene, NF-kappaB RelA phosphorylation regulates RelA acetylation, Mol. Cell. Biol. 25 (2005) 7966–7975.
[44] H.L. Pahl, Activators and target genes of Rel/NF-kappaB transcription factors, Oncogene 18 (1999) 6853–6866.
[45] P.J. Barnes, M. Karin, Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases, N. Engl. J. Med. 336 (1997) 1066–1071.
[46] K. Goto, M. Watanabe, H. Kondo, H. Yuasa, F. Sakane, H. Kanoh, Gene cloning, sequence, expression and in situ localization of 80 kDa diacylglycerol kinase specific to oligodendrocyte of rat brain, Brain Res. Mol. Brain Res. 16 (1992) 75–87.
[47] M.A. Sanjuan, D.R. Jones, M. Izquierdo, I. Merida, Role of diacylglycerol kinase alpha in the attenuation of receptor signaling, J. Cell Biol. 153 (2001) 207–220.
[48] X.P. Zhong, E.A. Hainey, B.A. Olenchock, H. Zhao, M.K. Topham, G.A. Koretzky, Regulation of T cell receptor-induced activation of the Ras-ERK pathway by diacylglycerol kinase zeta, J. Biol. Chem. 277 (2002) 31089–31098.
[49] Y. Shirai, S. Segawa, M. Kuriyama, K. Goto, N. Sakai, N. Saito, Subtype-specific translocation of diacylglycerol kinase alpha and gamma and its correlation with protein kinase C, J. Biol. Chem. 275 (2000) 24760–24766.
[50] A. Cipres, S. Carrasco, E. Merino, E. Diaz, U.M. Krishna, J.R. Falck, A.C. Martinez, I. Merida, Regulation of diacylglycerol kinase alpha by phosphoinositide 3-kinase lipid products, J. Biol. Chem. 278 (2003) 35629–35635.
[51] C.D. Nelson, S.J. Perry, D.S. Regier, S.M. Prescott, M.K. Topham, R.J. Lefkowitz, Targeting of diacylglycerol degradation to M1 muscarinic receptors by beta-arrestins, Science 315 (2007) 663–666.
[52] H.S. Mun, F. Aosai, K. Norose, M. Chen, L.X. Piao, O. Takeuchi, S. Akira, H. Ishikura, A. Yano, TLR2 as an essential molecule for protective immunity against *Toxoplasma gondii* infection, Int. Immunol. 15 (2003) 1081–1087.
[53] F. Yarovinsky, D. Zhang, J.F. Andersen, G.L. Bannenberg, C.N. Serhan, M.S. Hayden, S. Hieny, F.S. Sutterwala, R.A. Flavell, S. Ghosh, A. Sher, TLR11 activation of dendritic cells by a protozoan profilin-like protein, Science 308 (2005) 1626–1629.
[54] L.A. Lieberman, C.A. Hunter, The role of cytokines and their signaling pathways in the regulation of immunity to *Toxoplasma gondii*, Int. Rev. Immunol. 21 (2002) 373–403.
[55] C.A. Scanga, J. Aliberti, D. Jankovic, F. Tilloy, S. Bennouna, E.Y. Denkers, R. Medzhitov, A. Sher, Cutting edge: MyD88 is required for resistance to *Toxoplasma gondii* infection and regulates parasite-induced IL-12 production by dendritic cells, J. Immunol. 168 (2002) 5997–6001.
[56] K. Yanagisawa, S. Yasuda, M. Kai, S. Imai, K. Yamada, T. Yamashita, K. Jimbow, H. Kanoh, F. Sakane, Diacylglycerol kinase alpha suppresses tumor necrosis factor-alpha-induced apoptosis of human melanoma cells through NF-kappaB activation, Biochim. Biophys. Acta 1771 (2007) 462–474.
[57] M. Kai, S. Yasuda, S. Imai, M. Toyota, H. Kanoh, F. Sakane, Diacylglycerol kinase alpha enhances protein kinase Czeta-dependent phosphorylation at Ser311 of p65/RelA subunit of nuclear factor-kappaB, FEBS Lett. 583 (2009) 3265–3268.
[58] H. Zhong, H. SuYang, H. Erdjument-Bromage, P. Tempst, S. Ghosh, The transcriptional activity of NF-kappaB is regulated by the IkappaB-associated PKAc subunit through a cyclic AMP-independent mechanism, Cell 89 (1997) 413–424.
[59] H. Zhong, R.E. Voll, S. Ghosh, Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300, Mol. Cell 1 (1998) 661–671.
[60] A. Duran, M.T. Diaz-Meco, J. Moscat, Essential role of RelA Ser311 phosphorylation by zetaPKC in NF-kappaB transcriptional activation, EMBO J. 22 (2003) 3910–3918.
[61] I. Mattioli, H. Geng, A. Sebald, M. Hodel, C. Bucher, M. Kracht, M.L. Schmitz, Inducible phosphorylation of NF-kappa B p65 at serine 468 by T cell costimulation is mediated by IKK epsilon, J. Biol. Chem. 281 (2006) 6175–6183.
[62] D. Wang, A.S. Baldwin Jr., Activation of nuclear factor-kappaB-dependent transcription by tumor necrosis factor-alpha is mediated through phosphorylation of RelA/p65 on serine 529, J. Biol. Chem. 273 (1998) 29411–29416.
[63] H. Sakurai, H. Chiba, H. Miyoshi, T. Sugita, W. Toriumi, IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain, J. Biol. Chem. 274 (1999) 30353–30356.
[64] C. Limatola, D. Schaap, W.H. Moolenaar, W.J. van Blitterswijk, Phosphatidic acid activation of protein kinase C-zeta overexpressed in COS cells: comparison with other protein kinase C isotypes and other acidic lipids, Biochem. J. 304 (Pt 3) (1994) 1001–1008.
[65] R.F. Schwabe, H. Sakurai, IKKbeta phosphorylates p65 at S468 in transactivaton domain 2, FASEB J. 19 (2005) 1758–1760.
[66] L. Vermeulen, G. De Wilde, P. Van Damme, W. Vanden Berghe, G. Haegeman, Transcriptional activation of the NF-kappaB p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1), EMBO J. 22 (2003) 1313–1324.
[67] G.A. Webster, N.D. Perkins, Transcriptional cross talk between NF-kappaB and p53, Mol. Cell. Biol. 19 (1999) 3485–3495.
[68] R. Ravi, B. Mookerjee, Y. van Hensbergen, G.C. Bedi, A. Giordano, W.S. El-Deiry, E.J. Fuchs, A. Bedi, p53-Mediated repression of nuclear factor-kappaB RelA via the transcriptional integrator p300, Cancer Res. 58 (1998) 4531–4536.
[69] K.H. Vousden, Activation of the p53 tumor suppressor protein, Biochim. Biophys. Acta 1602 (2002) 47–59.
[70] A.S. Baldwin, Control of oncogenesis and cancer therapy resistance by the transcription factor NF-kappaB, J. Clin. Invest. 107 (2001) 241–246.
[71] W.C. Huang, T.K. Ju, M.C. Hung, C.C. Chen, Phosphorylation of CBP by IKKalpha promotes cell growth by switching the binding preference of CBP from p53 to NF-kappaB, Mol. Cell 26 (2007) 75–87.
[72] T. Tanaka, M. Okada, Y. Hozumi, K. Tachibana, C. Kitanaka, Y. Hamamoto, A.M. Martelli, M.K. Topham, M. Iino, K. Goto, Cytoplasmic localization of DGKzeta exerts a protective effect against p53-mediated cytotoxicity, J. Cell Sci. 126 (2013) 2785–2797.
[73] K. Goto, T. Tanaka, T. Nakano, M. Okada, Y. Hozumi, M.K. Topham, A.M. Martelli, DGKzeta under stress conditions: "to be nuclear or cytoplasmic, that is the question", Adv. Biol. Regul. 54 (2014) 242–253.
[74] N. Shikama, The p300/CBP family: integrating signals with transcription factors and chromatin, Trends Cell Biol. 7 (1997) 230–236.
[75] M.V. Karamouzis, P.A. Konstantinopoulos, A.G. Papavassiliou, Roles of CREB-binding protein (CBP)/p300 in respiratory epithelium tumorigenesis, Cell Res. 17 (2007) 324–332.
[76] H.M. Chan, N.B. La Thangue, p300/CBP proteins: HATs for transcriptional bridges and scaffolds, J. Cell Sci. 114 (2001) 2363–2373.
[77] H. Nakano, M. Shindo, S. Sakon, S. Nishinaka, M. Mihara, H. Yagita, K. Okumura, Differential regulation of IkappaB kinase alpha and beta by two upstream kinases, NF-kappaB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 3537–3542.
[78] Q. Zhao, F.S. Lee, Mitogen-activated protein kinase/ERK kinase kinases 2 and 3 activate nuclear factor-kappaB through IkappaB kinase-alpha and IkappaB kinase-beta, J. Biol. Chem. 274 (1999) 8355–8358.
[79] J. Ninomiya-Tsuji, K. Kishimoto, A. Hiyama, J. Inoue, Z. Cao, K. Matsumoto, The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway, Nature 398 (1999) 252–256.
[80] L.V. Madrid, C.Y. Wang, D.C. Guttridge, A.J. Schottelius, A.S. Baldwin Jr., M.W. Mayo, Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB, Mol. Cell. Biol. 20 (2000) 1626–1638.